PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 9/00, 9/22	A1	**(11) International Publication Number:** WO 96/22365 **(43) International Publication Date:** 25 July 1996 (25.07.96)

(21) International Application Number: PCT/US96/00711

(22) International Filing Date: 18 January 1996 (18.01.96)

(30) Priority Data:
08/380,671 20 January 1995 (20.01.95) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/380,671 (CON)
Filed on 20 January 1995 (20.01.95)

(71) Applicant *(for all designated States except US)*: THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL [US/US]; 300 Bynum Hall, Campus Box 4100, Chapel Hill, NC 27599-4100 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: TOPAL, Michael, D. [US/US]; 104 Craborchard Place, Chapel Hill, NC 27514 (US).

(74) Agents: SIBLEY, Kenneth, D. et al.; Bell, Seltzer, Park & Gibson, P.O. Drawer 34009, Charlotte, NC 28234 (US).

(81) Designated States: AL, AM, AT, AT (Utility model), AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AZ, BY, KG, KZ, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: ENZYMES THAT CLEAVE AND LIGATE DNA

(57) Abstract

Enzymes useful for carrying out the cleavage and ligation of DNA are disclosed that: (a) specifically bind to a DNA recognition site, (b) cleave the DNA on binding to the DNA recognition site to form a cleaved DNA having a cleaved end segment, (c) form a covalent intermediate with the cleaved DNA, (d) ligate the cleaved end segment with another end segment to form a ligated DNA, and (e) release the DNA from the covalent intermediate upon the formation of the ligated DNA. Nucleotides encoding the enzymes and methods of using the enzymes *in vitro* and *in vivo* are also disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

ENZYMES THAT CLEAVE AND LIGATE DNA

Field of the Invention

This invention concerns enzymes that exhibit both cleaving and ligating activity, recombining DNA as well as relaxing supercoiled DNA to give topoisomers thereof.

Background of the Invention

The topoisomerases, recombinases, and endonucleases share the ability to cleave DNA without sharing sequence homology. These enzymes catalyze DNA relaxation by cleavage, strand passage and reunion; catalyze DNA rearrangements by concerted cleavage and exchange of DNA ends; and catalyze cleavage of single- and double-stranded DNA, respectively. These enzymes are ubiquitous and have essential roles in the replication, transcription, recombination, and repair of DNA. *See, e.g.*, A. Kornberg and T.A. Baker, *DNA Replication* (2nd ed., W.H. Freeman and Co., New York, (1991)).

Topoisomerases function *in vivo* to both cleave and ligate supercoiled DNA and thereby relax the DNA. Topoisomerases demonstrate sequence preferences, but are not sequence specific, and hence are not generally useful in recombinant DNA techniques.

The endonucleases include as a special class the restriction enzymes, which function in bacteria to protect against foreign DNA. The restriction enzymes, along with the site-specific recombinases, offer examples of sequence-specific DNA cleavage. Additionally, restriction enzymes may function at some level *in vivo* to produce DNA with recombinogenic ends. *See, e.g.*, R.J. Roberts, *Crit. Rev. Biochem.* **4**, 123 (1976); W. Arber, *J. Struct. Biol.* **104**, 107 (1990); S. Chang and S.N. Cohen, *Proc. Natl. Acad. Sci.* USA **74**, 4811 (1977). The introduction of restriction enzymes into yeast and

mammalian cells has been shown to induce DNA rearrangements. R. Schiestl and T. Petes, *Proc. Natl. Acad. Sci.* USA **88**, 7585 (1991), E. Abella-Columna *et al.*, *Environ. Mol. Mutag.* **22**:26 (1993). These rearrangements may arise through random ligation of DNA fragments made recombinogenic by restriction endonuclease cleavage (S. Chang and S.N. Cohen, *Proc. Natl. Acad. Sci.* USA **74**:4811 (1977)).

Restriction enzymes and enzymes that ligate DNA (ligases) are together useful for carrying out recombinant DNA procedures. *See, e.g.*, S. Cohen and H. Boyer, U.S. Patent No. 4,740,470. Single enzymes that have both restriction activity and ligation activity in a site-specific manner would be extremely useful in recombinant DNA procedures *in vitro* and *in vivo*. However, enzymes with such an activity have not previously been known.

Summary of the Invention

A first aspect of the invention is an enzyme useful for carrying out the cleavage and rearrangement of DNA. The enzyme *(a)* specifically binds to a DNA recognition site; *(b)* cleaves the DNA on binding to the DNA recognition site to form a cleaved DNA having a cleaved end segment; *(c)* forms a covalent intermediate with the cleaved DNA; *(d)* ligates the cleaved end segment with another end segment to form a ligated DNA; and *(e)* releases the DNA from the covalent intermediate upon the formation of the ligated DNA.

A second aspect of the present invention is a polynucleotide encoding an enzyme as given above, along with host cells containing the polynucleotide and host cells that contain the polynucleotide and express the encoded enzyme.

A third aspect of the present invention is a method for cleaving and ligating DNA. The method comprises contacting a DNA to an enzyme as described

above, where the DNA contains a recognition site to which the enzyme specifically binds. The contacting step may be carried out *in vitro* or *in vivo*.

The foregoing and other objects and aspects of the present invention are explained in detail in the drawings herein and the specification set forth below.

Brief Description of the Drawings

Figure 1 shows the effect of the L43K mutation on the activity of *NaeI*; and

Figure 2 shows an electron micrograph visualization of a pBR322 dimer formed by the action of *NaeI*-L43K.

Figure 3 shows the reaction of *Nae I*-L43K with pBR322 (4 *Nae I* recognition sequences), M13 ds DNA (1 recognition sequence) and pUC (0 recognition sequences).

Detailed Description of the Invention

Amino acid sequences disclosed herein are presented in the amino to carboxy direction, from left to right. The amino and carboxy groups are not presented in the sequence. Both single letter and three letter abbreviations for amino acids are used.

Nucleotide sequences are presented herein by single strand only, in the 5' to 3' direction, from left to right.

The phrase "specifically binds" means binds one DNA segment of at least three bases out of the large pool of background DNA segments in a DNA population in a selective or preferential manner.

The phrase "recognition site" means the one DNA segment to which a particular enzyme specifically binds.

Site-specific cleavage of DNA is cleavage either within or nearby a recognition site. By "nearby" is meant within a defined or specific number of nucleotides from the recognition site.

A. Enzymes

Enzymes of the present invention specifically bind to a DNA recognition site and cleave the DNA on binding to the DNA recognition site to form a cleaved DNA having a cleaved end segment.

Enzymes of the present invention form a covalent intermediate with the cleaved DNA, ligate the cleaved end segment with another end segment to form a ligated DNA, and release the DNA from the covalent intermediate upon the formation of the ligated DNA. In a preferred embodiment of the invention, cleavage of the DNA is carried out in a site-specific manner, as noted above.

In one embodiment of the invention, where release of the DNA from the enzyme is concurrent with the formation of the ligated DNA, the covalent intermediate provides energy for the ligation reaction. Hence, the enzyme is not ATP-dependent, and in a particular embodiment is not ATP-dependent, is not NAD-dependent, and is not GTP-dependent. Alternatively, the covalent intermediate formed for the cleavage function of the enzyme may be released prior to the formation of the ligated DNA. In this case the covalent intermediate does not provide energy for the ligation reaction, and energy for the ligation reaction is provided by a ATP, NAD, GTP, or some other suitable energy source.

The specific binding activity of enzymes of the present invention is reflected in at least a 10^3-fold preference of the enzyme for the recognition site as compared to other segments, and may even be at least a 10^4-fold preference, at least a 10^6-fold preference, at least a 10^8-fold preference or more. The recognition site for the enzyme is, as noted above, at least three nucleotides in length, and may be 6, 8, 10, 12, 14, 16 or 18 nucleotides in length, or more. As will be appreciated by those skilled in the art, the presence of "STAR" sites, or sites of relaxed specificity, is common

for enzymes that specifically bind to DNA, and is not to be excluded from the present invention.

Enzymes according to the invention may be made from modification of an existing enzyme, including but not limited to type IIe restriction endonucleases, by techniques such as site-directed mutagenesis. The type IIe restriction enzymes require the recognition of a second DNA (effector) site to cleave DNA; putative homology has been found between the type IIE enzyme *Eco RII* and the integrase family of proteins. *See*, M. Topal and M. Conrad, *Nucleic Acids Res.* **21**:2599 (1993). Examples of type IIe enzymes include, but are not limited to, *Nae I* (M. Conrad and M. Topal, *Proc. Natl. Acad. Sci.* U.S.A. **86**:9707 (1989)), *Nar I*, *Bsp MI*, *Hpa II*, *Sac II* (A.R. Oller, *et al.*, *Biochem* **30**:2543 (1991), *Eco RII* (D.H. Kruger, *et al.*, *Nucl. Acids Res.* **16**:3997 (1988); S. Gabbara and A.S. Bhagwat, *J. Biol. Chem.* **267**:18623 (1992), *Atu BI*, *Cfr 9I*, *Sau BMKI*, *Eco 57I*, and *Ksp 632I* (M. Reuter et al., *Anal. Biochem.* **209**:232 (1993)). Thus, the type IIe enzymes are present in a wide variety of bacterial species.

Site-directed mutagenesis may be used to modify either the specificity (*i.e.*, change the recognition site) or activity of another enzyme, including but not limited to type IIe restriction enzymes, to produce enzymes of the instant invention. For example, the recognition site binding segment of one enzyme can be modified by substitution mutations to match the recognition site binding segment of a second enzyme to change the binding specificity of the first enzyme to that of the second enzyme. Site-directed mutagenesis may be carried out in accordance with known techniques. *See*, e.g., U.S. Patent No. 4,873,192 to Kunkel; T. Kunkel, *Proc. Natl. Acad. Sci. USA* **82**, 488 (1985); T. Kunkel et al., *Methods in Enzymol.* **154**, (1987). Commercially available kits for carrying out this technique may be used, such as the MUTA-GENE™ phagemid *in vitro*

mutagenesis kit by BIO-RAD (see generally BIO-RAD catalog number 170-3576 instruction manual).

The recognition site binding segment from one enzyme can be grafted into a second enzyme or exchanged with the recognition site binding segment of a second enzyme to produce enzymes of the present invention with a multiplicity of different binding specificities. Techniques for such grafting are discribed in, for example, A. Pessi et al., *Nature* **362**, 367 (1994), and such grafting techniques are well known in conjunction with the production of chimeric antibodies. *See, e.g.*, H. Waldmann, PCT Application WO93/01289.

Specificities of enzyme recognition sites may be altered by mutation, amplification and selection techniques such as the "SELEX" technique to produce enzymes of the present invention with altered binding and/or cleaving specificities. *See* U.S. Patent No. 5,270,163; *see also* C. Tuerck and L. Gold, *Science* **249**, 505-510 (1990).

Enzymes of the present invention with altered binding and/or cleaving specificities may be produced by phage display techniques in accordance with known techniques. *See, e.g.*, Y. Choo and A. Klug, *Proc. Natl. Acad. Sci. USA* **91**, 11163 (1994); J. Dejarlais and J. Berg, *Proc. Natl. Acad. Sci. USA* **90**, 2256 (1993); W. Huse et al., Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda, *Science* **246**, 1275 (1989).

Genetic engineering techniques for the production of enzymes of the present invention are discussed in greater detail below.

B. Genetic Engineering Techniques

A further aspect of the present invention is, as noted above, a polynucleotide such as a DNA encoding an enzyme as described herein. Such a polynucleotide may

be combined with a vector polynucleotide to provide a recombinant polynucleotide.

The production of cloned genes, recombinant DNA, vectors, transformed host cells, proteins and protein fragments by genetic engineering is well known. See, e.g., U.S. Patent No. 4,761,371 to Bell et al. at Col. 6 line 3 to Col. 9 line 65; U.S. Patent No. 4,877,729 to Clark et al. at Col. 4 line 38 to Col. 7 line 6; U.S. Patent No. 4,912,038 to Schilling at Col. 3 line 26 to Col. 14 line 12; and U.S. Patent No. 4,879,224 to Wallner at Col. 6 line 8 to Col. 8 line 59.

A vector or vector polynucleotide is a replicable polynucleotide construct. Vectors are used herein either to amplify DNA encoding an enzyme of the invention and/or to express the enzyme. An expression vector is a replicable polynucleotide construct in which a polynucleotide encoding an enzyme of the invention is operably linked to suitable control sequences capable of effecting the expression of the enzyme in a suitable host. The need for such control sequences will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation.

Amplification vectors do not require expression control domains. All that is needed is the ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants.

Vectors comprise plasmids, viruses (e.g., adenovirus, cytomegalovirus), retroviruses, phage, and integratable DNA fragments (i.e., fragments integratable into the host genome by recombination). The vector replicates and functions independently of the host

genome, or may, in some instances, integrate into the genome itself.

Expression vectors should contain a promoter and RNA binding sites which are operably linked to the gene to be expressed and are operable in the host organism.

DNA regions are operably linked or operably associated when they are functionally related to each other. For example, a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

Transformed host cells are cells which have been transformed or transfected with vectors containing a polynucleotide encoding an enzyme of the invention constructed using recombinant DNA techniques. Transformed host cells ordinarily express the enzyme, but host cells transformed for purposes of cloning or amplifying DNA need not express the enzyme.

Suitable host cells include prokaryote, yeast or higher eukaryotic cells such as mammalian cells and insect cells. Cells derived from multicellular organisms are also a suitable host for synthesis of the enzymes of the instant invention receptor by recombinant means. Propagation of such cells in cell culture has become a routine procedure (Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)). Examples of useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and WI138, BHK, COS-7, CV, and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located upstream from the polynucleotide encoding the enzyme of the invention to be expressed and operatively associated therewith, along with a ribosome binding site, an RNA splice site (if intron-containing

genomic DNA is used), a polyadenylation site, and a transcriptional termination sequence.

The transcriptional and translational control sequences in expression vectors to be used in transforming vertebrate cells are often provided by viral sources. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and Simian Virus 40 (SV40). See, e.g., U.S. Patent No. 4,599,308.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV 40 or other viral (e.g. Polyoma, Adenovirus, VSV, or BPV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

Rather than using vectors which contain viral origins of replication, one can transform mammalian cells by the method of cotransformation with a selectable marker and polynucleotides encoding enzymes of the instant invention. Examples of suitable selectable markers are dihydrofolate reductase (DHFR) or thymidine kinase. This method is further described in U.S. Pat. No. 4,399,216 (Applicant specifically intends that the disclosure of all patent references cited herein be incorporated herein by reference).

Other methods suitable for adaptation to the expression of an enzyme of the invention in recombinant vertebrate cell culture include those described in M-J. Gething et al., Nature 293, 620 (1981); N. Mantei et al., Nature 281, 40; A. Levinson et al., EPO Application Nos. 117,060A and 117,058A.

Host cells such as insect cells (e.g., cultured *Spodoptera frugiperda* cells) and expression vectors such as the baculovirus expression vector (e.g., vectors derived from *Autographa californica* MNPV, *Trichoplusia ni* MNPV, *Rachiplusia ou* MNPV, or *Galleria ou* MNPV) may be employed in carrying out the present invention, as

described in U.S. Patents Nos. 4,745,051 and 4,879,236 to Smith et al. In general, a baculovirus expression vector comprises a baculovirus genome containing the gene to be expressed inserted into the polyhedrin gene at a position ranging from the polyhedrin transcriptional start signal to the ATG start site and under the transcriptional control of a baculovirus polyhedrin promoter.

Prokaryote host cells include gram negative or gram positive organisms, for example Escherichia coli (E. coli) or Bacilli. Higher eukaryotic cells include established cell lines of mammalian origin as described below. Exemplary host cells are E. coli W3110 (ATCC 27,325), E. coli B, E. coli X1776 (ATCC 31,537), E. coli 294 (ATCC 31,446). A broad variety of suitable prokaryotic and microbial vectors are available. E. coli is typically transformed using pBR322. Promoters most commonly used in recombinant microbial expression vectors include the beta-lactamase (penicillinase) and lactose promoter systems (Chang et al., Nature 275, 615 (1978); and Goeddel et al., Nature 281, 544 (1979)), a tryptophan (trp) promoter system (Goeddel et al., Nucleic Acids Res. 8, 4057 (1980) and EPO App. Publ. No. 36,776) and the tac promoter (H. De Boer et al., Proc. Natl. Acad. Sci. USA 80, 21 (1983)). The promoter and Shine-Dalgarno sequence (for prokaryotic host expression) are operably linked to the polynucleotide of the invention i.e., they are positioned so as to promote transcription of messenger RNA from the DNA.

Eukaryotic microbes such as yeast cultures may also be transformed with vectors carrying polynucleotides of the invention. see, e.g., U.S. Patent No. 4,745,057. Saccharomyces cerevisiae is the most commonly used among lower eukaryotic host microorganisms, although a number of other strains are commonly available. Yeast vectors may contain an origin of replication from the 2 micron yeast plasmid or an autonomously replicating sequence (ARS), a promoter, a DNA encoding an enzyme of the

invention, sequences for polyadenylation and transcription termination, and a selection gene. An exemplary plasmid is YRp7, (Stinchcomb et al., Nature 282, 39 (1979); Kingsman et al., Gene 7, 141 (1979); Tschemper et al., Gene 10, 157 (1980)). Suitable promoting sequences in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem. 255, 2073 (1980) or other glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg. 7, 149 (1968); and Holland et al., Biochemistry 17, 4900 (1978)). Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., EPO Publn. No. 73,657.

Enzymes of the invention produced as described above may be isolated and/or purified in accordance with conventional techniques, such as affinity chromatography, gel filtration, ultrafiltration, size-exclusion chromatography, etc.

C. Compositions.

The nucleotides and enzymes of the present invention include the salts thereof, including physiologically and/or pharmaceutically acceptable salts thereof: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto. Examples of such salts are (a) salts formed with cations such as sodium, potassium, NH_4^+, magnesium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid,

methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

Compositions or formulations of the present invention comprise the active agent (*e.g.*, enzyme, polynucleotide encoding the enzyme, vector carrying the polynucleotide, etc.) in a physiologically or pharmaceutically acceptable carrier, such as an aqueous carrier. In the manufacture of a formulation according to the invention, the active agent is typically admixed with, *inter alia*, an acceptable carrier.

In the composition or formulation the active agent (*e.g.*, the enzyme of the invention) may be contained within a lipid particle or vesicle, such as a liposome or microcrystal, which may be suitable for parenteral administration. The particles may be of any suitable structure, such as unilamellar or plurilamellar, so long as the enzyme is contained therein. Positively charged lipids such as N-[1-(2,3-dioleoyloxi)propyl]-N,N,N-trimethyl-amoniummethylsulfate, or "DOTAP," are particularly preferred for such particles and vesicles. The preparation of such lipid particles is well known. *See, e.g.*, U.S. Patents Nos. 4,880,635 to Janoff et al.; 4,906,477 to Kurono et al.; 4,911,928 to Wallach; 4,917,951 to Wallach; 4,920,016 to Allen et al.; 4,921,757 to Wheatley et al.; etc. Such compositions are particularly useful for delivering enzymes of the invention into a cell, as discussed below.

D. Applications of the Instant Invention

As will be apparent from the foregoing, enzymes of the present invention possess a variety of uses. They are useful for cleaving DNA, for relaxing supercoiled DNA, for specifically cleaving and recombining DNA *in vivo* or *in vitro*, etc. They are useful as molecular weight markers. In general, the enzymes of the invention

are useful as tools for carrying out genetic engineering and recombinant DNA techniques. In addition, DNA that is specifically cleaved and/or specifically ligated with the enzymes of the instant invention to produce a DNA of a particular molecular weight is useful as a molecular weight marker.

A method for cleaving and ligating DNA, comprises contacting a DNA to an enzyme as given herein, where the DNA contains a recognition site to which the enzyme specifically binds. The contacting step may be carried out so that only a single DNA species is cleaved and re-ligated, or may be carried out so that a first DNA species is cleaved and a second DNA species is ligated into the first DNA species to produce a recombinant DNA species. The contacting step may be carried out by any suitable means: it may be carried out *in vitro* in an aqueous solution; it may be carried out *in vivo* in a cell. Where carried out *in vivo*, the cell may be one that contains a polynucleotide encoding the enzyme and expresses the encoded enzyme. Alternatively, the enzyme may be an exogeneous enzyme introduced into the cell by any suitable means, such as providing the enzyme contained within a lipid vesicle such as a liposome, and contacting the lipid vesicle to the cell so that the contents thereof are transported into the cell and released therein.

The present invention is explained in greater detail in the following non-limiting Examples, where mg means milligrams, μg means micrograms, ng means nanograms, ml means milliliters, μl means microliters, mM means millimoles, ds means double stranded, kDA means kilodaltons, BSA means bovine serum albumin, SDS means sodium dodecyl sulphate, min means minutes, and temperatures are given in degrees Centigrade.

EXAMPLE 1

Site-Directed Mutagenesis of NaeI

Nae I is a 70 kDa dimeric protein with two DNA-binding sites, as indicated by the sigmoidal dependence of cleavage velocity on the concentration of recognition sequence. *See* C. Yang and M. Topal, *Biochem.* **31**:9657 (1992). *Nae I* is highly specific for its recognition sequence and exhibits a 10^{11}-fold discrimination between cognate and noncognate recognition sequences. *See*, C. Yang *et al.*, *Biochem.* **33**, 14918 (1994). The two DNA-binding sites of *Nae I* are nonidentical: one site prefers to bind to the recognition site with AT-rich flanking sequences, whereas the other prefers to bind to the recognition site with GC-rich flanking sequences (see Yang and Topal, *supra*). It is unclear whether the binding differences preexist or are induced upon occupation of one DNA binding site. Since *Nae I* must bind two DNA recognition sequences, a DNA substrate with a single *Nae I* recognition sequence. This resistance can be overcome by the introduction of another DNA recognition sequence, either in cis or in trans, with affinity for the second DNA-binding site on the enzyme. *See*, M. Conrad and M. Topal, *Proc. Natl. Acad. Sci. USA* **86**:9707 (1989).

Nae I also induces loops in pBR322 DNA with *Nae I* bound at the base of the loops. M. Topal *et al.*, *Biochem.* **30**, 2006 (1991). Comparisons of the *Nae I* amino-acid sequence with amino-acid sequences of the recombinase, transposase, and topoisomerase families failed to detect similarities by computer searching with a basic local alignment search tool. *See* S. Altschul *et al.*, *J. Mol. Biol.* **215**, 403 (1990). When the amino-acid sequence of *Nae I* was compared with that of the DNA ligases, another ubiquitous family of proteins used by the same basic biological processes to seal DNA breaks, a ten amino-acid region, near the NH_2 terminus of the

putative *Nae I* active site, was found to match the consensus for the active site of DNA ligase I **(TABLE 1)**.

TABLE 1. Comparison of an amino terminal region of NaeI with the active-site region for enzyme-adenylate formation in DNA ligases.

Type	Sequence	SEQ ID NO:
NaeI	39 TLDQLYDGQR 48	SEQ ID NO:1
Human	564 TCEYKYDGQR 573	SEQ ID NO:2
S. Pombe	412 TCEYKYDGER 421	SEQ ID NO:3
S. cerevisiae	416 TSEYKYDGER 425	SEQ ID NO:4
Vaccinia	227 FAEVKYDGER 236	SEQ ID NO:5
T7	30 IAEIKYDGVR 39	SEQ ID NO:6
T3	30 IADCKYDGVR 39	SEQ ID NO:7
T4	156 FAQLKADGAR 165	SEQ ID NO:8
E. coli	111 CCELKLDGLA 120	SEQ ID NO:9
T. thermophilus	114 TVEHKVDGLS 123	SEQ ID NO:10

The better matches of *Nae I* are with the active sites of the eukaryotic rather than the prokaryotic DNA ligases; the best match is with that of human DNA ligase I. The *Nae I* sequence differs, however, from the human ligase active site in one important respect: The lysine (K) that forms the adenylated intermediate essential for catalysis by the DNA ligase active site, is not present in *Nae I*, instead there is a leucine (L43) at this position.

T to A mutations of both nucleotides 127 and 128 (counting from the first initiating methionine) was carried out by site-directed mutageneisis in accordance with standard techniques.

In the enzyme, the two mutations carried out by site-directed mutageneis changed L43 to K43 and concomitantly changed *Nae I* activity. Thus, this single amino-acid change gave *Nae I* classic topoisomerase

activity, which requires DNA cleavage, strand passage, and religation.

EXAMPLE 2

Activity of *Nae I*-L43K

Site-directed mutagenesis changed L43 to K43 and concomitantly changed *Nae I* activity. Products were resolved by electrophoresis through 1% agarose gels and staining with ethidium bromide. Reactions used 1 ng of purified *Nae I* with 0.1 μg pBR322 DNA, 30 ng of purified *Nae I*-L43K with 1 μg pBR322 DNA, and 1 μg (total protein) of the respective cell extracts (not shown) and 1 μg pBR322 DNA in 15 μl of reaction buffer [10 mM Tris-Cl (pH 8.0), 20 mM Nacl, 10 mM $MgCl_2$, BSA (0.1 mg/ml), 5.0 mM β-mercaptoethanol] for 50 min at 37°C.

Figure 1 illustrates the effect of the L43K mutation on the activity of *Nae I*. **Figure 1** shows the products of incubating *Nae I* wild-type (wt) and *Nae I*-L43K with pBR322 DNA; lane 3, incubated with commercial *Drosophila* topoisomerase II (USB); lane 4, incubated with purified *Nae I*-L43K.

Incubation of pBR322 DNA, which contains four *Nae I* recognition sites, with reaction-limiting amounts of wt *Nae I* resulted in a partial digest (**Fig. 1**, lanes 1 and 2). Incubation of pBR322 DNA with *Nae I*-L43K resulted in relaxation of the supercoiled DNA in a step-wise manner to yield covalently-closed DNA topoisomers (**Fig. 1**, lane 4). The bimodal distribution of superhelical and relaxed DNA molecules implies a predominantly processive mode of action under these reaction conditions (H. Vosberg., *Current Topics in Microbiol. and Immun.* **114**:19 (1985)), meaning several enzyme cycles before dissociation. The relaxation by *Nae I*-L43K was identical to that caused by commercial topoisomerase (**Fig. 1**, lane 3). After the reaction had gone to completion, half of the supercoiled DNA substrate had been converted by *Nae I*-L43K to relaxed covalently

closed DNA; the other half was nicked (data not shown). Thus, this single amino-acid change gave *Nae I* classic topoisomerase activity, which requires DNA cleavage, strand passage, and religation.

Cell extracts without the overexpressed *Nae I*-L43K mutant or that overexpress *Nae I*-E70K, a variant that binds to but cannot cleave DNA (J. Holtz and M.D. Topal, *J. Biol. Chem.* **269**, 27286 (1994)), lacked the topoisomerase activity. Thus, the enriched topoisomerase activity is specific to the L43K variant.

EXAMPLE 3

Endonuclease and ligase domain activity of *Nae I*-L43K

The apparent activities of the endonuclease and ligase domains of *Nae I*-L43K were observed by the effect of *Nae I*-L43K on completely relaxed covalently-closed circular DNA. The use of completely relaxed DNA eliminates topoisomers as possible substrates. Such DNA was prepared by cleaving and religating pBR322 DNA at the unique Hind III site. *Nae I*-L43K cleaved this DNA substrate to convert the closed-circle to full-length linear products and used both the cleavage and ligation functions to produce higher-molecular-weight species (data not shown). The products from this reaction, and from reactions in which *Nae I*-L43K was allowed to completely relax supercoiled pBR322 DNA, were found by electron microscopy to contain double-length circular and linear molecules as well as the expected unit length circles and cleaved products in proportions consistent with the proportions of the major species resolved by gel electrophoresis.

Figure 2 shows the visualization of a pBR322 dimer formed by action of *Nae I*-L43K. Monomer pBR322 DNA circles (smaller circle) were incubated with *Nae I*. Reaction products were treated with SDS to remove protein and purified by gel permeation chromatography. DNA samples were prepared by EM using the denatured protein

monolayer method. Molecular lengths were determined by measurements taken directly from micrographs using a Summagraphics digitizer. The circle crossing at its center is a dimer. EM courtesy of Dr. Kyusung Park, UNC. Bar equals 0.5 micrometers.

EXAMPLE 4

Effect of ATP on Nae-I-L43K activity

The *Nae I*-L43K activity was isolated and purified to apparent homogeneity.

To purify the enzyme, cells are cracked open and debris spun out to produce a cell-free extract in accordance with standard techniques. The cell-free extract is then bound to DEAE-cellulose and eluted with buffer (20 mM potassium phosphate, pH 6.9, 0.1 mM EDTA, 1 mM β-mercaptoethanol, and 5% glycerol) containing a 0.05 to 1.0 M salt gradient. The DEAE fraction in the eluate containing overexpressed topoisomerase activity were dialyzed, bound to an S-sepharose column, and eluted with buffer containing a 0.05 M salt gradient. The appropriate S-sepharose fraction in this second eluate was then in turn dialyzed, bound to phosphocellulose, and eluted with buffer containing a 0.1 to 0.6 M salt gradient. The appropriate S-sepharose fraction in this third eluate was then in turn dialyzed, bound to heparin agarose, and eluted with buffer containing a 0.1 to 1M salt gradient. The resulting protein in the final eluate was greater than 97% pure and was purified to apparent homogeneity (when run on a standard polyacrylamide gel electrophoresis gel with SDS buffer, a single band is identified by Coomassie blue staining (30 μg protein per lane).

All known ligases require ATP or NAD for activity. *See, e.g.*, T. Lindahl and D. Barnes, *Annu. Rev. Biochem.* **61**, 251 (1992). Purified *Nae I*-L43K, however, processively reduced the linking number of pBR322 DNA (**Fig. 1**) and produced higher-molecular-weight

products (data not shown) without an outside energy source: ATP, GTP, and NAD had no measurable effect on *Nae I*-L43K activity. Moreover, an adenylated intermediate was not detected when using *Nae I*-L43K with either radiolabeled ATP or NAD, under conditions that gave this intermediate using both bacteriophage T4 DNA ligase with labeled-ATP, and *E. coli* DNA ligase with labeled-NAD. Thus, the ligase motif in *Nae I* does not act as a classic ATP or NAD ligase.

This independence from an outside energy source implied that either *Nae I*-L43K alone or both *Nae I*-L43K and wild type *Nae I* (which just performs the cleavage step), form activated covalent protein-DNA intermediates. An activated intermediate was implicated by the DNA relaxation activity of *Nae I*-L43K because energy is required for ligation, but the cleavage and ligation steps in DNA relaxation are interrupted by a DNA strand-passage step. Such covalent intermediates are common energy sources for strand breakage and reunion among the topoisomerases (J. Wang, *Annu. Rev. Biochem.* **54**:665 (1985)) and recombinases (N. Craig, *Annu. Rev. Genet.* **22**:77 (1988)). To observe this intermediate for *Nae I* and *Nae I*-143K, we used heat to interrupt the reaction with radiolabeled DNA substrate. Extensive nuclease treatment of the reaction transferred the radioactive label from DNA substrate to protein: the nuclease-treated, radiolabeled *Nae I*-DNA complex survived boiling in SDS and gave a denatured protein band by SDS-PAGE with an apparent molecular weight consistent with that of *Nae I*.

Purified *Nae I* (0.47 μM) was incubated with 300 ng of pBR322 DNA in 15 μl of reaction buffer described in Example 1 above. The reaction was stopped after 30 min at 37°C by heating to 70°C for 10 min. The reaction products were digested with exonuclease III and DNase I overnight, then made 2% in SDS and 5% in β-mercaptoethanol and boiled for 3 min. The reaction

products were then resolved by SDS-PAGE (10% acrylamide gel/4% spacer gel, 0.4% SDS) and visualized by autoradiography. In addition, *Nae I* protein was denatured by boiling in SDS, electrophoresed, and located by Coomassie blue staining. Prestained molecular weight markers (carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase B, and myosin heavy-chain) were also run and apparent molecular weights, as reported by the manufacturer, were also examined.

A similar result was found using *Nae I*-L43K, but much less labeled protein was observed. The more transient nature of the intermediate in the L43K mutant compared to that in wt *Nae I* is consistent with a concerted displacement of the protein during religation.

The mechanism of displacement of the activated *Nae I*-DNA intermediate appears to be analogous to that in DNA ligases. The covalent intermediate formed between wt *Nae I* and pBR322 DNA indicate that the activated intermediate is formed during the cleavage step, independent of the L43K mutation. Thus, analogous to the use by unadenylated DNA ligase of an activated DNA-AMP intermediate for sealing DNA breaks, the unadenylated ligase motif of *Nae I*-L43K may use the activated DNA-protein intermediate formed during the cleavage step for resealing DNA breaks.

EXAMPLE 5

Site-specific binding of Nae-I-L43K

Different DNAs were tested as substrates for *Nae I*-L43K as a test for sequence discrimination. Plasmid pUC18 lacks as *Nae I* recognition sequence; M13 double-strand DNA has one such sequence that is resistant to cleavage by *Nae I* because of its poor affinity for the *Nae I* effector-binding site. *See* M. Conrad and M. Topal, *Proc. Natl. Acad. Sci.* U.S.A. **86**, 9707 (1989); C. Yang and M. Topal, *Biochem*, **31**, 9657 (1992). Neither of these DNAs were substrates for *Nae I*-L43K. Plasmid pBR322 has

four *Nae I* recognition sequences and is a good substrate for wt *Nae I*; this plasmid was a good substrate for *Nae I*-L43K (**Fig. 3**).

Figure 3 shows the reaction of *Nae I*-L43K with pBR322 (4 *Nae I* recognition sequences), M13 ds DNA (1 recognition sequence) and pUC (0 recognition sequences). Purified *Nae I*-L43K (30 ng) (shown) and cell extract (1 μg total protein) (not shown) was incubated with; Lanes 1 to 3, pBR322 DNA (1.0 μg); Lanes 4 to 6, M13mp18 DNA (0.5 μg); Lanes 7 to 9, pUC DNA (0.75 μg) under reaction conditions indicated in the legend to Fig. 2. Products from all reactions were resolved by gel electrophoresis (1% agarose) and visualized by staining with ethidium bromide.

These results indicate that the topoisomerase activity of *Nae I*-L43K is sequence dependent and demonstrate the relationship of *Nae I*-L43K topoisomerase activity to *Nae I* protein. The sequence dependency of *Nae I*-L43K demonstrates that the protein conserves at lease one of the two DNA binding sites even as it gains additional ligase function.

The foregoing Examples are illustrative of the present invention, and are not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Topal, Michael D.

(ii) TITLE OF INVENTION: Enzymes that Cleave and Ligate DNA

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Kenneth D. Sibley; Bell, Seltzer, Park and Gibson
(B) STREET: Post Office Drawer 34009
(C) CITY: Charlotte
(D) STATE: North Carolina
(E) COUNTRY: U.S.A.
(F) ZIP: 28234

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Sibley, Kenneth D.
(B) REGISTRATION NUMBER: 31,665
(C) REFERENCE/DOCKET NUMBER: 5470-99

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 919-420-2200
(B) TELEFAX: 919-881-3175
(C) TELEX: 575102

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Thr Leu Asp Gln Leu Tyr Asp Gly Gln Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Thr Cys Glu Tyr Lys Tyr Asp Gly Gln Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Thr Cys Glu Tyr Lys Tyr Asp Gly Glu Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Thr Ser Glu Tyr Lys Tyr Asp Gly Glu Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Phe Ala Glu Val Lys Tyr Asp Gly Glu Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ile Ala Glu Ile Lys Tyr Asp Gly Val Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ile Ala Asp Cys Lys Tyr Asp Gly Val Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Phe Ala Gln Leu Lys Ala Asp Gly Ala Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Cys Cys Glu Leu Lys Leu Asp Gly Leu Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Thr Val Glu His Lys Val Asp Gly Leu Ser
1 5 10

THAT WHICH IS CLAIMED IS:

1. An enzyme useful for carrying out the cleavage and rearrangement of DNA, which enzyme:

specifically binds to a DNA recognition site;

cleaves said DNA on binding to said DNA recognition site to form a cleaved DNA having a cleaved end segment;

forms a covalent intermediate with said cleaved DNA;

ligates said cleaved end segment with another end segment to form a ligated DNA; and

releases said DNA from said covalent intermediate upon the formation of said ligated DNA.

2. An enzyme according to claim 1, wherein said enzyme is not ATP-dependent.

3. An enzyme according to claim 1, wherein said enzyme is not ATP-dependent, is not NAD-dependent, and is not GTP-dependent.

4. An enzyme according to claim 1, wherein said cleavage of said DNA is carried out in a site-specific manner.

5. An enzyme according to claim 1, wherein said recognition site is at least three nucleotides in length.

6. An enzyme according to claim 1, wherein said enzyme is an isolated enzyme.

7. An enzyme according to claim 1, wherein said enzyme is a type IIe restriction endonuclease.

8. An enzyme according to claim 1, wherein said enzyme is an *NaeI* restriction endonuclease.

9. An enzyme according to claim 1, wherein said enzyme is an *NaeI* restriction endonuclease having leucine to lysine substitution mutation at amino acid position 43.

10. An enzyme according to claim 1, wherein said enzyme is contained within a lipid vesicle.

11. A polynucleotide encoding an enzyme useful for carrying out the cleavage and rearrangement of DNA, which enzyme:

specifically binds to a DNA recognition site;

cleaves said DNA on binding to said DNA recognition site to form a cleaved DNA having a cleaved end segment;

forms a covalent intermediate with said cleaved DNA;

ligates said cleaved end segment with another end segment to form a ligated DNA; and

releases said DNA from said covalent intermediate upon the formation of said ligated DNA.

12. A polynucleotide according to claim 11, wherein said polynucleotide is a DNA.

13. A recombinant polynucleotide comprising vector polynucleotide and a polynucleotide according to claim 11.

14. A cell containing a polynucleotide according to claim 11.

15. A cell containing a polynucleotide according to claim 11, and wherein said cell expresses said enzyme.

16. A method for cleaving and ligating DNA, comprising:

contacting a DNA to an enzyme according to claim 1, said DNA containing a recognition site to which said enzyme specifically binds.

17. A method according to claim 16, wherein said contacting step is carried out *in vitro* in an aqueous solution.

18. A method according to claim 16, wherein said contacting step is carried out *in vivo* in a cell that containing a polynucleotide according to encoding said enzyme, and wherein said cell expresses said enzyme.

19. A method according to claim 16, wherein said contacting step is carried out *in vivo* in a cell, and wherein said enzyme is an exogeneous enzyme introduced into said cell.

20. A method according to claim 19, wherein said enzyme is contained within a lipid vesicle.

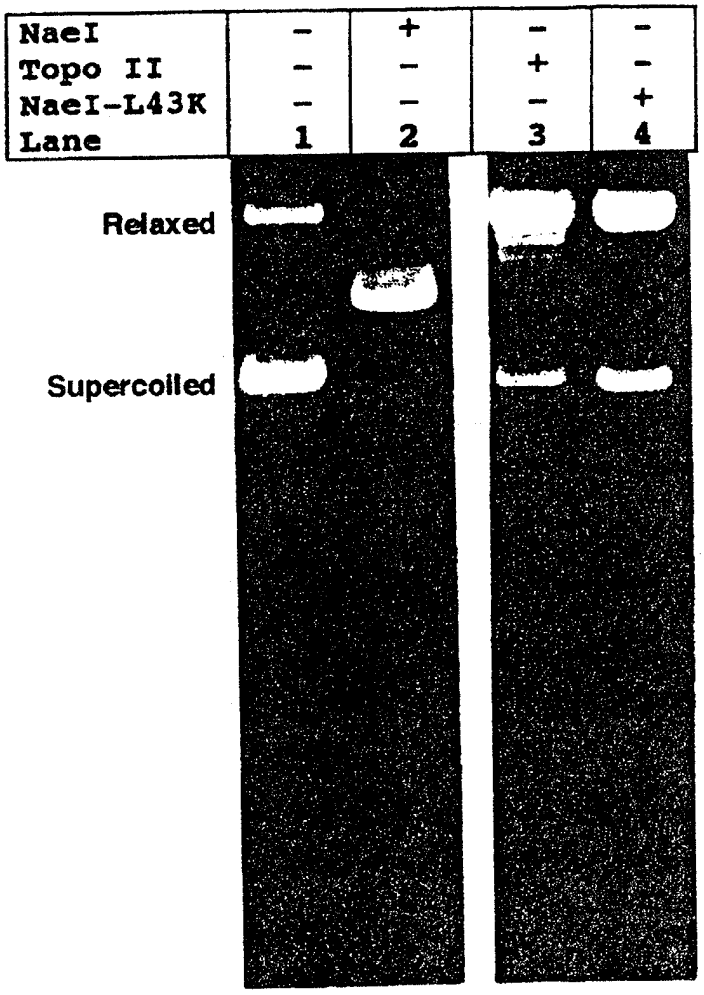

FIG. 1

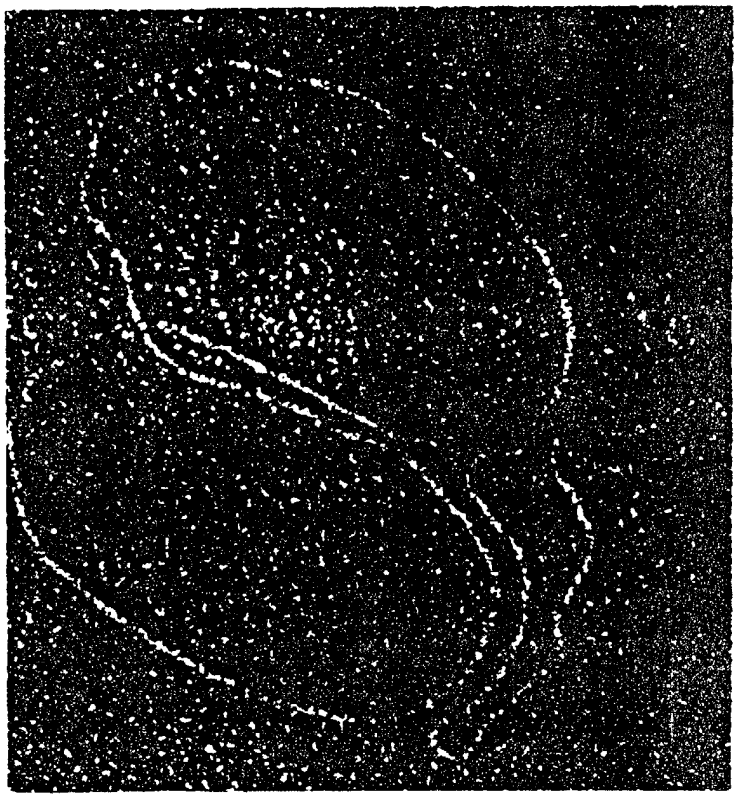

FIG.2

NaeI-L43K	-	-	+	-	-	+	-	-	+
Dros. topo II	-	+	-	-	-	-	-	-	+
pBR322 DNA	+	-	+	-	-	+	-	-	+
M13 RF DNA	-	+	+	-	-	-	+	-	+
pUC18 DNA	-	-	-	+	-	+	-	+	-
Lane	1	2	3	4	5	6	7	8	9

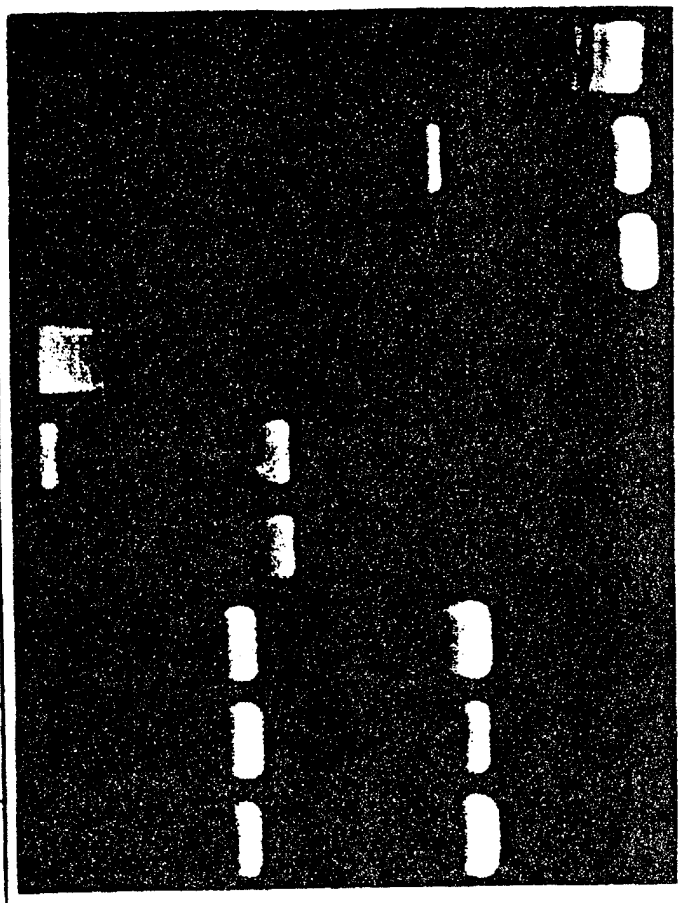

FIG.3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/00711

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 9/00, 9/22
US CL :435/183, 199

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/183, 199

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	Science, Vol. 267, issued 24 March 1995, Jo et al., "DNA topoisomerase and recombinase activities in Nae I restriction endonuclease", pages 1817-1820, see the entire document.	1-10
X	Nature, Vol. 327, issued 18 June 1987, Busk et al., "Preferential relaxation of supercoiled DNA containing a hexadecameric recognition sequence for topoisomerase I", pages 638-640, see the entire document.	1-6
X	Journal of Biological Chemistry, Vol. 269, No. 51, issued 23 December 1994, Shuman, "Novel approach to molecular cloning and polynucleotide synthesis using vaccinia DNA topoisomerase", pages 32678-32684, see the entire document.	1-6

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 APRIL 1996	Date of mailing of the international search report 26 APR 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ERIC GRIMES Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/00711

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	US, A, 5,434,066 (BEBEE ET AL.) 18 July 1995, see the entire document.	1-6, 10
Y	Journal of Molecular Biology, Vol. 181, issued 1985, Hoess et al., "Mechanism of strand cleavage and exchange in the Cre-*lox* site-specific recombination system", pages 351-362, see the entire document.	1-6, 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/00711

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-10

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/00711

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, Dialog
search terms: ligase, ligate, recombinase, topoisomerase, endonuclease, restriction enzyme, mutate, mutation, mutant, mutagenesis

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claims 1-10, drawn to a DNA cleaving and ligating enzyme.

Group II, claims 11-15, drawn to DNA encoding a DNA cleaving and ligating enzyme, and vectors and host cells comprising said DNA.

Group III, claims 16-20, drawn to a process of enzymatically cleaving and ligating DNA.

The inventions listed as Groups I to III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The claims of Group I share a technical feature of an enzyme that site-specifically cleaves and ligates DNA. The claims of Group II share a technical feature of DNA encoding an enzyme the site-specifically cleaves and ligates DNA. The claims of Group III share a technical feature of using an enzyme to site-specifically cleave and ligate DNA.
Enzymes that site-specifically cleave and ligate DNA, as well as DNA encoding such enzymes and methods of using such enzymes, were known in the prior art. See, e.g., Shuman et al., J. Biol. Chem. 269:32678 (1994); Busk et al., Nature 327:638 (1987); and Bebee et al., U.S. Patent 5,434,066. The various Groups of inventions thus do not share a technical relationship involving one or more of the same or corresponding special technical features, i.e., those technical features that define a contribution which each claimed invention, considered as a whole, makes over the prior art. They therefore do not fulfill the requirements of unity of invention and a holding of lack of unity for examination purposes is proper.